

NEUTROPHILS INFLUENCE MELANOMA ADHESION AND MIGRATION UNDER FLOW CONDITIONS

Margaret J. SLATTERY and Cheng DONG*

Department of Bioengineering and The Huck Institute for Life Sciences, The Pennsylvania State University, University Park, PA, USA

We have studied human melanoma cell (C8161) adhesion and migration in response to stimulation by soluble collagen IV (CIV) using a modified Boyden chamber. In this modified chamber, shear flow can be introduced over the cell-substrate interface, affecting tumor cell chemotactic migration through a microporous filter. A relatively high level of intercellular adhesion molecule-1 (ICAM-1) was found on C8161 cells. In contrast, levels of β_2 -integrins (e.g., LFA-1 and Mac-1), the molecules that would be necessary for C8161 stable adhesion to the endothelium substrate, were found to be very low on these melanoma cells. As a result, C8161 trans-endothelial migration under a flow condition of 4 dyn/cm² decreased by 70% as compared to static migration. When human neutrophils (PMNs) were present in the tumor cell suspension, C8161 migration recovered by 85% over C8161 cells alone under the 4 dyn/cm² flow condition. Blocking ICAM-1 on C8161 cells or Mac-1 on PMNs significantly inhibited C8161-PMN adhesion and subsequent C8161 migration through the endothelium under flow conditions. In addition, increased interleukin-8 production and Mac-1 expression by PMNs were detected when they were co-cultured with C8161 melanoma cells. These results suggest that transmigration of C8161 cells under flow conditions can be influenced by PMNs, mediated by Mac-1/ICAM-1 adhesive interactions and enhanced by altered cytokine production.

© 2003 Wiley-Liss, Inc.

Key words: *in vitro* extravasation; shear stress; type IV collagen; interleukin-8; Mac-1/ICAM-1

The initial step in melanoma extravasation is tumor cell adhesion to the endothelium of capillaries. However, the cellular and molecular mechanisms responsible for regulating melanoma adhesion are still poorly defined. It has become evident from *in vivo* studies^{1–4} that the mechanisms utilized by leukocytes and metastatic tumor cells to adhere to a vessel wall prior to extravasation are very different.

Human leukocytes, including neutrophils (PMNs), actively participate in the inflammatory response *via* adhesion to the vascular endothelium.^{5–7} Significant progress has been made in the past decades toward understanding how PMNs roll along the endothelium before forming shear-resistant bonds. Detailed studies of cell-cell interactions suggest that the selectins are required for the initial rolling of leukocytes on activated endothelium.^{8,9} Whereas the much stronger binding, responsible for prolonged shear-resistant attachment, is mediated by β_2 -integrins (Mac-1 or LFA-1) expressed on the leukocyte and ICAM-1 (intercellular adhesion molecule-1) on the endothelial cells.¹⁰ Several studies have suggested that LFA-1 to ICAM-1 adhesion is important in initial endothelial capture of PMNs, while Mac-1 and ICAM-1 interaction forms shear-resistant bonds to stabilize PMN-endothelium adhesion.^{11,12} The question of whether or not these mechanisms apply to tumor cells remains.

How do tumor cells bind to the endothelium? One observation from *in vivo* video microscopy has indicated that tumor cells can be trapped in capillaries and only arrest on the endothelium on the basis of vessel-size restriction in the microcirculation of whatever organ or tissue they extravasate.¹ In contrast, another *in vivo* study has discovered that the B16F1 melanoma cells could become arrested by shear flow-resistant adhesion to the walls of presinusoidal vessels in mice pretreated with the cytokine Interleukin-1 α (IL-1 α).³ Clearly, those studies are somewhat contradictory and

additional work is required to characterize the event of tumor cell-endothelium adhesion.

In general, either the host immune response or extreme hemodynamic forces will destroy tumor cells before they have any chance to attach to a blood vessel surface. However, there are examples of tumor cells exploiting leukocytes and enhancing binding and metastasis.^{13,14} Human PMNs, which comprise 50–70% of circulating leukocytes and are usually cytotoxic to tumor cells, have been shown under certain circumstances to promote tumor adhesion and transendothelial migration.¹⁵ A role for PMNs in metastasis was suggested by a study that showed that PMNs and activated macrophages increased the ability of rat hepatocarcinoma cells to adhere to an endothelial monolayer.¹⁶ Furthermore, tumor-elicited PMNs, in contrast to normal PMNs, were found to enhance metastatic potential and invasiveness of rat mammary adenocarcinoma cells in an *in vivo* tumor-bearing rat model.¹⁴ Using light and electron microscopy, circulating PMNs were discovered in close association with metastatic tumor cells including at the time of tumor cell arrest.¹⁷ Although these studies suggest that PMNs can enhance tumor cell adhesion, there is little understanding of the mechanisms potentially involved.

Melanoma cells have been found to produce many chemokines. Two chemokines of particular interest are monocyte chemotactic and activating factor (MCAF) and interleukin-8 (IL-8). Soluble MCAF (also known as monocyte chemotactic protein MCP-1/CCL2) has been shown to augment monocyte cytostatic activity.¹⁸ The second, soluble IL-8, possesses neutrophil chemotactic and activating capacities^{19,20} in addition to T-lymphocyte chemotactic activity.²¹ Although several pro-inflammatory cytokines and chemokines have been implicated in influencing adhesive properties of transformed cells, IL-8 is of particular interest. In addition to activating and recruiting leukocytes at sites of inflammation, IL-8 promotes the growth of some tumors and production is associated with metastatic potential.²²

The advancement of experimental assays to characterize cellular adhesion and migration are in a period of rapid development.^{23–26} For example, PMN-endothelium adhesion has been widely examined using various *in vitro* experimental systems such as the parallel-plate flow chamber whereas leukocyte and tumor cell migration has been studied using the Boyden-chemotaxis chamber.

Grant sponsor: National Institutes of Health; Grant number: CA-76434; Grant sponsor: National Institutes of Health; Grant number: CA97306; Grant sponsor: National Science Foundation; Grant number: BES-138474; Grant number: Penn State University Life Science Consortium; Grant number: Seed.

*Correspondence to: Department of Bioengineering 229 Hollowell Building, The Pennsylvania State University, University Park, PA 16802. Fax: +001-814-863-0490. E-mail: cxd23@psu.edu

Received 25 September 2002; Revised 23 January 2003, 25 April 2003; Accepted 28 April 2003

DOI 10.1002/ijc.11297

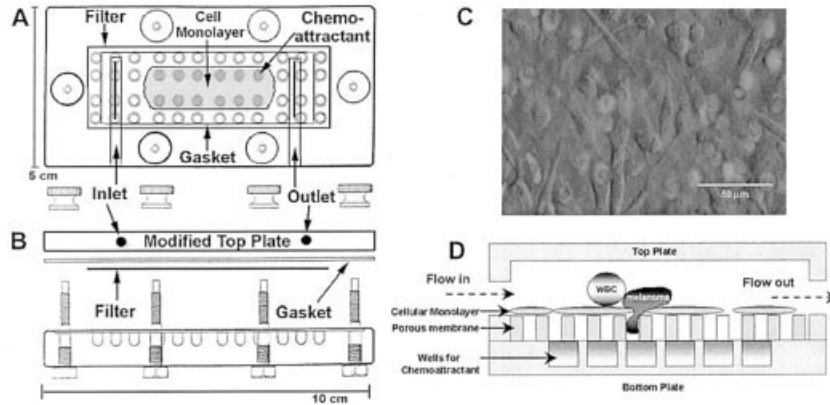


FIGURE 1 – Flow migration chamber from the top (a) and the side views (b). The top plate and bottom chemotactic wells were machined from polycarbonate. The gasket is silicone and filter is a polycarbonate membrane with 8 μm pores. (c) Micrograph of an EI cell monolayer that was grown on the filter. EI cells provide a consistent cell substrate with a stable level of ICAM-1 expression. (d) Circulating flow provides a model of a microvascular environment where a cell monolayer acts as a barrier that cells must migrate through in response to chemotactic stimulation from the bottom wells.

However, the limitation of these assays is that each is restricted to modeling either only adhesion under flow conditions or migration under static conditions and does not permit investigating how dynamic flow conditions alter cell extravasation. One motivation of our study is to develop a new approach to studying tumor cell adhesion and migration using a novel *in vitro* flow-migration assay,²⁶ which will allow us to investigate cellular and molecular mechanism of PMN-mediated melanoma cell extravasation under dynamic shear-flow conditions.

MATERIAL AND METHODS

Cell culture

C8161.c9 melanoma cells (provided by Dr. D. Welch, Penn State Medical Center) were maintained as described previously.²⁷ C8161 cells were also transfected to constitutively express green fluorescent protein (GFP) and maintained as described elsewhere.²⁸ C8161 cells were prepared in both stimulated and unstimulated cases. Stimulated C8161 cells were treated with IL-8 (10 ng/ml, 1 hr) (R&D System, Minneapolis, MN), Tumor Necrosis Factor- α (TNF- α ; 10U/ml, 24 hr) (R&D System) or Phorbol 12-myristate 13-acetate (PMA; 100 ng/ml, 20 min) (Sigma Chemical Co., St. Louis, MO) to specifically upregulate ICAM-1, Mac-1 or LFA-1, respectively. C8161 cells were treated with antibodies by incubating 1×10^6 cells with 5 μg of IgG anti-human ICAM-1 (CalTag Laboratories, South San Francisco, CA) or IgG anti-GFP (Sigma Chemical Co.) in blocking buffer (PBS with 5% calf serum and 2% goat serum) for 30 min at 37°C. Prior to each experiment, C8161 cells were detached when nearly confluent and suspended in fresh medium and allowed to recover for 1 hr while being rocked at a rate of 8 times per minute (rpm) at 37°C. The cells were then resuspended to a concentration of 1×10^6 cells/ml in serum free DMEM-F12 supplemented with 0.1% w/v BSA (Sigma Chemical Co.) and allowed to rock (8 rpm) at 37°C for an additional hour.

Neonatal melanocytes (NHEM; Cambrex-Clonetics, E. Rutherford, NJ) cells were maintained in manufacturer's EGM-2-MV medium. Prior to each experiment the cells were detached and rocked (8 rpm) for 1 hr in culture medium at 37°C and then for an additional hour in RPMI 1640 with 0.1% w/v BSA. The viability of detached NHEM cells has been tested; approximately 80% of the detached NHEM cells were found to be viable both immediately after detachment and after 2 hr of preparatory rocking.

Fibroblast L-cells that had been transfected to express human ICAM-1 (EI cells; provided by Dr. S. Simon, UC Davis) were maintained in culture as described elsewhere.³⁴ ICAM-1 levels on EI cells were shown to be comparable with IL-1 α stimulated human umbilical vein endothelial cells (HUVEC)²⁹ and were used as a substrate for cell adhesion and a model endothelial cell in our study. To block ICAM-1, EI monolayers were incubated in blocking buffer with 10 μg of IgG anti-human ICAM (CalTag Laboratories) at 37°C for 30 min. The cell monolayer was then gently

washed with warmed media prior to the experiment to remove excess antibody.

Neutrophil isolation

Fresh blood was obtained from healthy adults under informed consent as approved by The Pennsylvania State University IRB. Neutrophils (PMNs) were isolated and enriched using Ficoll-Hypaque gradient (Sigma Chemical Co.). The isolated PMN layer was first suspended in 0.1% human serum albumin (HSA; Sigma Chemical Co.) in DPBS and washed. The cell pellet was then suspended in ACK lysis buffer (0.15 M NH_4Cl , 10.0 mM KHCO_3 and 0.1 mM Na_2EDTA in distilled H_2O) for 5 min to remove red cells. Finally, the cells were washed and resuspended in 0.1% HSA/DPBS at a concentration of 1×10^6 cells/ml. The PMNs were rocked (8 rpm) at 37°C until they were assayed, which was usually within 2 hr. To activate cells, PMNs were also treated with IL-8 (10 ng/ml, 1 hr). Mac-1 was functionally blocked by treating the cells with 5 μg of IgG anti-human Mac-1 (CalTag Laboratories) per 1×10^6 cells in blocking buffer for 30 min at 37°C. Fixed cells were prepared by suspending cells in 4% paraformaldehyde and incubated at 4°C for 1 hr. The cells were then washed and resuspended in media in a concentration appropriate for the experiment. Cell death was verified using trypan blue.

Flow-migration chamber assay

The *in vitro* flow-migration device is a modified 48-well chemotactic Boyden chamber that has been recently developed (Fig. 1).²⁶ In brief, the polycarbonate chamber consists of a top and bottom plate separated by a gasket. The bottom plate has 48 centered chemotactic wells and screws around the perimeter to affix the top plate. The top plate has an inlet and outlet allowing for circulating flow through the chamber. The gasket is an 11 cm \times 5.5 cm piece of 0.02 inch-thick silicon (SFMedical, Hudson, MA), in which a 7 cm \times 2 cm opening was cut from the center for the flow field. The wall shear stress (τ_w) is related to the volumetric flow rate (Q) by $\tau_w = 6\mu Q/w(h^2)$, where μ is the fluid viscosity, h is height and w is width of the flow field. PVP-free polycarbonate filters (8 μm pore size; NeuroProbe, Gaithersburg, MD) were sterilized and coated with fibronectin (30 $\mu\text{g}/\text{ml}$, 3 hr) (BD Biosciences, Lexington, KY). Prior to each experiment, a monolayer of EI cells was grown nearly to 100% confluence on prepared filters (typically 36–48 hr after seeding with cells).

The chamber's center 12 wells (Fig. 1) were filled with soluble chemoattractant, type IV collagen (CIV; 50 or 100 $\mu\text{g}/\text{ml}$ in RPMI 1640/0.1% BSA) (BD Biosciences)^{25,30,31} and control wells were filled with medium (RPMI 1640/0.1% BSA). The flow loop was primed with warmed medium to eliminate bubbles in the system. The chamber was assembled by pipetting the cells of interest (C8161 only; PMN only; or C8161+PMN together) into the flow field allowing the cells to presettle on the monolayer substrate for approximately 10 min. An equal number of each cell types was put in the chamber, 5×10^5 cells; in the case of C8161+PMN a total of

1×10^6 cells were put in the chamber. Finally, the fittings were tightened and the entire apparatus was placed in a 37°C , 5% CO_2 incubator. Initially a very slow flow (2 ml/min) was pumped through the system. The flow rate was then increased to the desired rate (0–20 ml/min). Experiments were run at a selected wall shear stress (0, 2 and 4 dyn/cm^2) for a predetermined length of time (1, 3 and 4 hr). When the assay was completed, the flow chamber was removed from the flow loop and disassembled and the filter was gently removed and imaged either by fluorescence microscopy and then stained with Protocol Brand Hema3 solution (Fisher Scientific, Pittsburgh, PA) or immediately stained. The cells on the bottom side of the filter were imaged using an inverted microscope and captured *via* NIH Image (v. $\beta 4.0.2$) on a PC. Three images of each migration filter were quantified and averaged for each data point. For each data point, at least 3 filters were analyzed. Random PMN and C8161 migration were subtracted from each sample and no C8161 cells were found in the chemoattractant wells after 4 hr of migration.

Statistical analysis of cell-migration data

Statistical significance between cases tested in the flow migration chamber was determined by the unpaired *t*-test on Sigma Plot (v. 8.0) with the corresponding *p* value reported for each case. All error bars on flow migration data histograms represent the standard error of the mean (SEM).

Adhesion assay

Cell-cell adhesion strength was measured by using the parallel-plate chamber assay. The glass substrate of the flow chamber had a confluent monolayer of EI cells. The cell suspension, prepared with fluorescein (FITC)-labeled C8161 cells with or without tetramethylrhodamine (TRITC)-stained PMNs, was perfused through the flow chamber until suspending cells reached the field of view and settled down to the chosen monolayer substrate. For the duration of an experiment, the flow was introduced in 10–20 sec intervals with 10 sec of rest between each stepwise increase in flow shear stresses. All experiments were recorded on videotape for analysis. The adherent cells of interest were counted and monitored after each increase in shear stress.

Flow cytometry

The cells of interest were prepared as described above and then treated with murine anti-human CD marker primary antibodies (*e.g.*, anti-LFA-1, anti-Mac-1 or anti-ICAM-1; $1 \mu\text{g Ab}/10^6$ cells) (CalTag Laboratories) for 30 min at 4°C . The cells were then treated with secondary antibody, FITC-conjugated goat anti-mouse IgG F(ab)₂ fragment ($1 \mu\text{g}/10^6$ cells) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 25 min at 4°C . The samples were fixed with 4% formaldehyde (Sigma Chemical Co.) and analyzed using Coulter EPICS XL (Coulter Corp., Hialeah, FL) flow cytometer. Control cases used to determine background fluorescence were samples treated with secondary antibody only.

Viability assays

Viability assays were performed according to manufacturer's suggested protocol (Molecular Probes, Eugene, OR; LIVE/DEAD Kit L-7013). Briefly, a stock solution of HEPES-buffered saline solution (HEPES-BSS) was made and supplemented with SYTO 10 and DEAD Red at a 1:500 dilution of each. Cells were cultured in 6-well plates, either alone (one cell type per well), in Transwell culture or in contact culture. In Transwell (Costar, Cambridge, MA) culture, C8161 cells were in the bottom well and PMNs were placed in the upper well. For Contact culture, both cell types were placed in a single well and where allowed to interact. Cell culture medium was removed from approximately 10^6 cells by centrifuging at 250g for 10 min. The cell pellet was resuspended in 200 μl of the dye supplemented HEPES-BSS and incubated for 15 min at room temperature, completely protected from light. After incubation, the cell suspension was centrifuged and the dye supernatant was aspirated. The cell pellet was then resuspended in 4% glutaraldehyde in HEPES-BSS and held at 4°C for 1 hr. After 1 hr, the

cells were pelleted again and the fixative was removed. The pellet was resuspended in HEPES-BSS and stored at 4°C protected from light until analysis by flow cytometry.

Protein detection and analysis (ELISA, immunoblot, and RT-PCR)

Enzyme-linked immunosorbent assay (ELISA) detection of IL-8 secretion was performed by the Penn State NIH Cytokine Core Facility. Samples were prepared from supernatant of cultured cells. Approximately 1×10^6 cells, as counted *via* hemacytometer, were cultured in fresh medium for 6 hr. All supernatant samples were spun at 500g for 3 min to remove debris and stored at -70°C prior to analysis. In the cases of PMN and C8161 cell co-culture, the 2 cell types were cultured in 6-well plate Transwells, where C8161 cells were adherent to the bottom wells while 1×10^6 PMNs were added in the $4 \mu\text{m}$ insert. The 2 cell types were not in contact during either the 6 hr (ELISA and RT-PCR samples) or 24 hr (Immunoblot samples) co-culture.

Whole cell extracts were also prepared from the cells co-cultured as described above. Cells (5×10^6) were suspended in 100 μl of $2 \times$ SDS running dye (0.2% bromophenol blue, 4% SDS, 100 mM Tris pH6.8, 2 mM DTT, 3 ng/ml aprotinin, 2 ng/ml pepstatin A, 1 ng/ml leupeptin, 0.8 mM PMSF, 1 mM AEBSF, 50 μM bestatin, 15 μM E-64 and 30% glycerol). Samples were loaded onto a 15% SDS-PAGE gel and the protein was transferred to a 0.2 μm nitrocellulose filter (Schleicher and Schuell, Keene, NH) by electroblotting. The filter was blocked for 1 hr in phosphate-buffered saline containing 0.2% Tween-20 (PBST) with 5% nonfat milk (NFM). IL-8 was detected by diluting mouse anti-IL-8 IgG1 (0.8 $\mu\text{g}/\text{ml}$; Biosource, Samarillo, CA) in PBST plus 5% NFM and incubating overnight at 4°C . Following incubation with the primary antibody, the filter was washed with PBST at room temperature $3 \times$ before adding the secondary antibody (peroxidase-conjugated goat anti-mouse IgG diluted 1:5,000 in PBST with 5% NFM). After 1 hr incubation, the filter was washed again in PBST $3 \times$ followed by a single wash in PBS. IL-8 was detected using the Enhanced Chemiluminescence Detection System (Amersham Biosciences, Arlington Heights, IL).

Cells co-cultured in Transwell inserts as described above were washed in cold PBS at 1.5×10^5 cells/ml and lysed with 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl and 0.1 M β -mercaptoethanol. RNA was then isolated by a phenol-chloroform extraction. The aqueous RNA fractions were treated with 10 units of DNase I for 20 min at 24°C to remove genomic DNA and purified with a second phenol-chloroform extraction. cDNA was prepared from 1 μg of RNA, using a murine leukemia virus reverse transcriptase and random primers. Samples that did not include reverse transcriptase (RT) served as controls for genomic DNA contamination. To amplify the cDNA, a 30-cycle polymerase chain reaction (PCR) was performed for 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min with 0. μg of cDNA using the target primers. Interleukin-8 cDNA was amplified using the primer pair 5'TCTGCAGCTCTGTGTGAAGG3' and 5'TA-ATTTCTGTGTTGGCGCAG3'. μ -actin cDNA was amplified using the primer pair 5'CCTAAGGCCAACCGTGAAAAG3' and 5'TCTTCATGGTGCTAGGAGCCA3'. The cDNA was separated by electrophoresis on a 5% polyacrylamide gel and stained with ethidium bromide for imaging.

RESULTS

C8161 melanoma cells express functional ICAM-1 but not β_2 -integrins

Flow cytometry was used to analyze surface expression of β_2 -integrins (*e.g.*, Mac-1 and LFA-1) and ICAM-1 on both unstimulated and stimulated C8161 cells. No significant LFA-1 or Mac-1 adhesion molecule expression was detected above background (Fig. 2a). No change in fluorescence level was found, as compared to the unstimulated cases, for LFA-1 or Mac-1 expression when C8161 cells were stimulated with PMA or IL-8, respec-

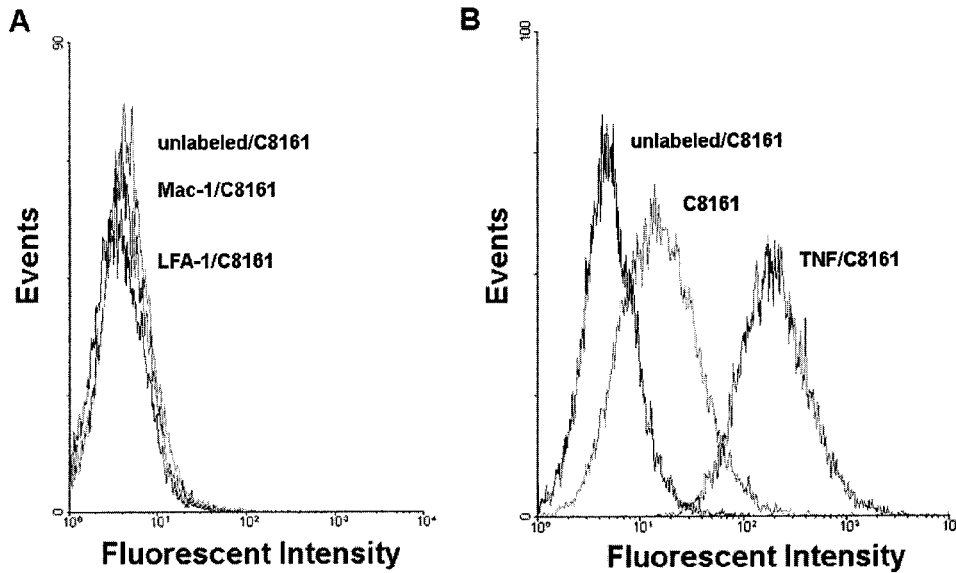


FIGURE 2—Expression of cell adhesion molecules on C8161 melanoma cells as detected by flow cytometry. Histogram of “unlabeled/C8161” samples served as a control for auto-fluorescence. (a) “Mac-1/C8161” and “LFA-1/C8161” are surface expression of Mac-1 and LFA-1 on unstimulated C8161 cells, respectively. Cytokine stimulation does not result in any significant increase in Mac-1 or LFA-1 on C8161 cells (data not shown). (b) ICAM-1 expression on TNF α -stimulated (TNF/C8161) and unstimulated C8161 cells (C8161).

tively (data not shown). This indicates C8161 cells do not express Mac-1 or LFA-1, which are most commonly found on leukocytes. In contrast, ICAM-1 expression on C8161 cells showed a significant shift in the fluorescence level from background and especially when the cells were stimulated with TNF- α , suggesting C8161 cells express significant levels of ICAM-1 molecules (Fig. 2b). The ICAM-1 expressed on C8161 cells was found to functionally bind β_2 -integrin ligands as tested with an adhesion assay (data not shown).

Neutrophils enhance C8161 migration under flow conditions

C8161 cell chemotactic migration in response to type IV collagen (CIV) (100 $\mu\text{g}/\text{ml}$) was characterized under both static and flow conditions (Fig. 3b). When exposed to a shear flow (4 dyn/cm^2), nearly 3.5 times fewer C8161 cells migrated toward CIV than those under the no flow condition (Fig. 3a). The static migration assays showed that C8161 chemotaxis was virtually the same in the presence or absence of PMNs (Fig. 3a; $p=0.47$). The addition of PMNs to the C8161 suspension significantly enhanced C8161 migration to CIV under shear stress (Fig. 3a; $p<0.01$). As negative controls, NHEM (melanocytes) migration was tested under the static condition and NHEM cells were co-suspended with PMNs using the flow-migration assay to test for cell migration under shear conditions. Melanocyte chemotaxis was found to be nearly the same as background level in each case (Fig. 3a). Fixed PMNs were used to account for the increase in cell number while not introducing biological affects. Results shown in Figure 3a indicate that when fixed PMNs are present, C8161 cells migrate at a similar level to C8161 cells alone, suggesting that doubling the cell number in the system would not change the cell migratory behavior.

Several control experiments were conducted to verify the flow-migration data. No C8161 cells were found in the chemoattractant wells after a 4 hr period of migration (data not shown), confirming our notion that C8161 cells remain adhered to the filter after migration.³⁰ To determine which cells had migrated. GFP/C8161 cells were used so that migrated C8161 could be differentiated from any possible migrated PMNs. GFP/C8161 cells were then subtracted from the total migrated cells to determine the number of PMNs that would migrate (Fig. 3b). The number of PMNs that had migrated with C8161 cells was not significantly different from the number of PMNs that had migrated on their own in response to CIV, at a negligible level.

A checkerboard-type study was performed to examine the chemokinesis of C8161 in the migration chamber. Four cases were

examined: 1) control media in the top and bottom well; 2) media in the top well, CIV in the bottom well; 3) CIV in both the top and bottom wells and 4) CIV in the top well and media in the bottom well. We found, as expected, migration only in the case where media was in the top well and CIV in the bottom well with a chemoattractant gradient (Fig. 3c). The 3 other cases showed no cell migration.

C8161 cell migration to CIV was also studied for 3 different durations of time (1, 3 and 4 hr; Fig. 4a). Very little C8161 cell chemotaxis (under 4 dyn/cm^2 shear stress) occurred at the 1 hr time point with or without PMNs. C8161 migration at the 3 or 4 hr time points was significantly increased compared to the 1 hr period, especially in the presence of PMN. Migration appeared to have plateaued (3 hr vs. 4 hr with PMN; $p=0.72$). Whereas in the absence of PMNs, C8161 migration over 4 hr showed a slight increase over that with a 3 hr period ($p=0.076$).

Two different CIV concentrations (50 and 100 $\mu\text{g}/\text{ml}$) were tested in the flow-migration chamber. At a lower CIV concentration, C8161 cells did not show significant migration toward CIV, with or without PMNs (Fig. 4b). At a higher CIV concentration, C8161 cells were able to migrate at an increased level without PMNs; however, PMNs significantly enhanced C8161 migration ($p=0.007$). These results show chemotactic gradient effects on melanoma cell migration, under the influence of PMNs.

C8161 cell migration under different shear stresses (0, 2 and 4 dyn/cm^2) was examined (Fig. 4c). A similar level of C8161 migration to CIV was found in the presence or absence of PMNs under static conditions, whereas under shear conditions (2 and 4 dyn/cm^2), PMNs significantly promoted C8161 chemotaxis ($p=0.003$ and 0.007, respectively) compared to those without PMNs.

PMN-mediated C8161 adhesion and migration is ICAM-1 dependent

Adhesion molecules involved in PMN-C8161 interaction were characterized using the flow migration chamber. Under the influence of IL-8-activated PMNs, TNF- α -stimulated C8161 cells resulted in higher chemotaxis than TNF- α -stimulated C8161 cells alone ($p>0.002$) (Fig. 5a). PMA-activated PMNs produced a less significant increase in C8161 chemotactic migration (Fig. 5a). This suggests that Mac-1 on PMNs may be involved in PMN-mediated C8161 adhesion and migration. To investigate ICAM-1 involvement in PMN-influenced C8161 migration, adhesion was tested by blocking ICAM-1 on C8161 cells with antibody treatment. Antibody (anti-ICAM-1)-treated C8161 cells migrated at a signifi-

cantly lower level than untreated C8161 cells in the presence of PMNs (Fig. 5b), exhibiting a similar level of migration of untreated C8161 cells in the absence of PMNs (Fig. 5a). These results suggest that PMNs could mediate C8161 cell adhesion *via* an ICAM-1-dependant mechanism, hence enhancing melanoma migration. To test this hypothesis, untreated C8161 cells were added to anti-Mac-1 treated PMNs. Blocking Mac-1 on PMNs reduced C8161 chemotactic migration (Fig. 5b). In addition, ICAM-1 molecules were blocked on the EI cell monolayer, preventing cell adhesion to the substrate. This even more dramatically reduced C8161 migration and brought it to nearly background levels. To test for nonspecific antibody blocking, C8161 cells treated with anti-GFP were found to migrate at the same level as untreated cells (Fig. 5b). Together, these results suggest PMN-

mediated C8161 adhesion and migration under flow conditions could be *via* an ICAM-1/Mac-1 dependent mechanism.

C8161 and PMN aggregation results in stronger C8161 adhesion to endothelium

A parallel-plate flow chamber was used to examine possible heterotypic cell aggregation between C8161 and PMNs, which could mediate C8161 cell adhesion to the EI monolayer under shear stress. As seen from Figure 6, TNF- α -stimulated C8161 cells that aggregated with IL-8-activated PMNs result in stronger tumor cell adhesion to an EI cell surface than unstimulated C8161-PMN pairs. TNF- α -stimulated C8161 cells were found to have similar adhesion characteristics as unstimulated C8161 cells. Antibody blocking either ICAM-1 on C8161 cells or Mac-1 on PMNs, respectively, reduced C8161-PMN aggregation and decreased C8161 adhesion to EI cell monolayer to a similar low level as C8161 cells alone without PMNs. This data suggests that improved cell adhesion to an ICAM-1 expressing monolayer is one of the primary mechanisms by which PMNs can influence C8161 migration.

Cell viability assessment

C8161 cells and PMNs were tested for viability in culture to determine if PMNs were cytotoxic to the C8161 cells. Three different culture conditions were assayed: 1) separate culture (single cell type); 2) Transwell culture (2 cell types noncontact coculture; data shown in Fig. 7a,b) and 3) contact culture (2 cell types). The cells were also tested at 3 different time intervals, 4, 20 and 24 hr of culture. Figure 7 shows representative dot plots of the cells as detected by flow cytometry. The x-axis is increasing "live-cell" green fluorescence, while the y-axis is a measure of red fluorescence, which stained dead cells. The lower right quadrant is the region where the live cells fall, while the upper right quadrant encompasses dead cells. Greater than 90% of the C8161 cells and PMNs cultured alone or in Transwells were found to be alive, for all time points. Viability of PMNs that were cultured in contact with C8161 cells for extended periods of time (20 or 24 hr) was significantly less than 90%. Therefore, PMNs are not cytotoxic to the melanoma cells during the time frame of the flow migration assay (approximately 4 hr).

C8161 cells increase IL-8 production in PMNs

The possibility of secreted chemokines as a stimulus responsible for cell-cell communication between melanoma cells and PMNs was examined. ELISA and Western blot were used to assess IL-8

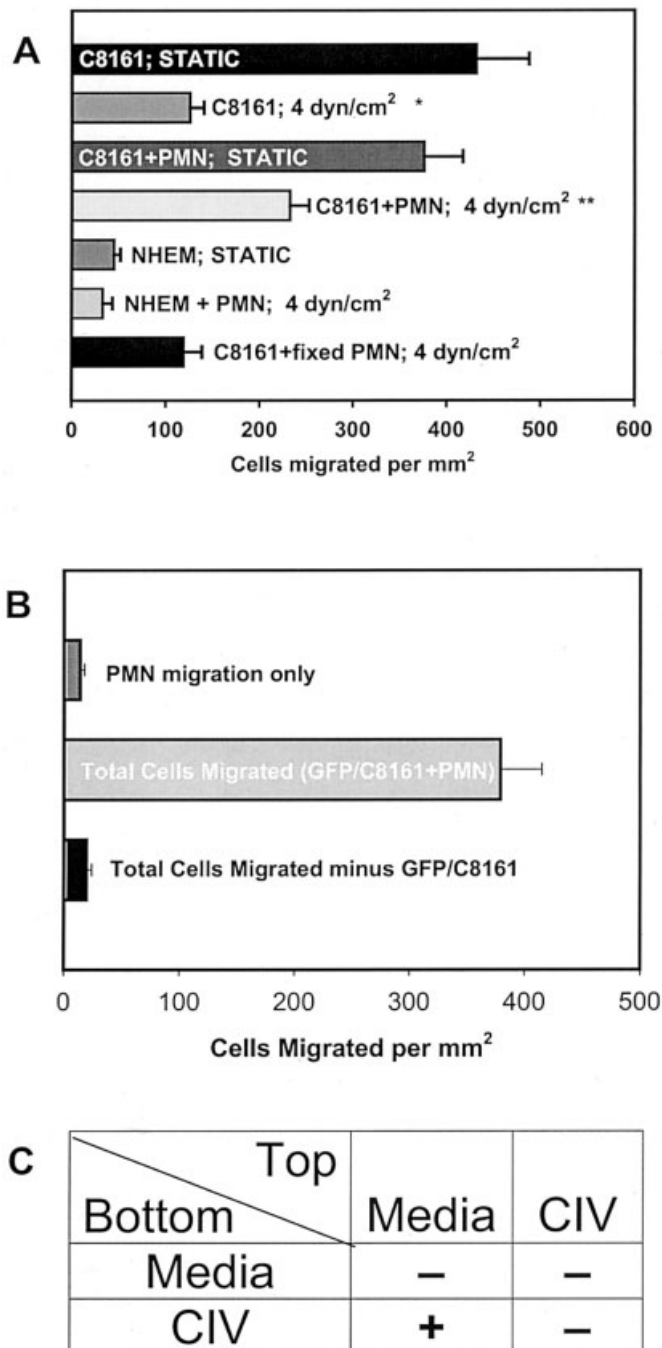


FIGURE 3 – (a) C8161 melanoma cell chemotactic migration to CIV (100 μ g/ml) over a 4 hr period. The number of migrated C8161 cells dramatically falls when the cells were exposed to 4 dyn/cm² of shear stress (C8161; 4dyn/cm²) compared to the migration under static condition (* p =0.002 with respect to "C8161; Static" case), while the presence of PMNs does not affect C8161 migration under static conditions (C8161+PMN; Static). C8161 cell migration is significantly higher in the presence of PMNs (C8161+PMN; 4dyn/cm²) than those C8161 cells without PMNs under flow conditions (* p <0.01 with respect to "C8161; 4dyn/cm²" case). Melanocytes (NHEM) were used as negative controls showing that noninvasive cells do not migrate toward CIV under static (NHEM; Static) or shear conditions even in the presence of PMN (NHEM+PMN; 4dyn/cm²). Fixed PMNs were also added to C8161 suspension. Results indicate adding nonfunctional cell numbers does not promote C8161 migration. (b) PMN migration toward CIV when tested with or without GFP/C8161 cells. The number of PMNs migrated in either case is very low and not significantly different, indicating that total cells migrated (as shown in Fig. 3a) are C8161 cells. GFP/C8161 were found to migrate at the same level as nontransfected C8161 cells (data not shown). (c) C8161 cells only migrate (+) in response to a gradient of type IV collagen created by placing media in the top well and CIV in the bottom well. C8161 cell migration was not found (-) when CIV was in both top and bottom wells, in top well only or not present. All results were obtained under 100 μ g/ml CIV and over 4 hr.

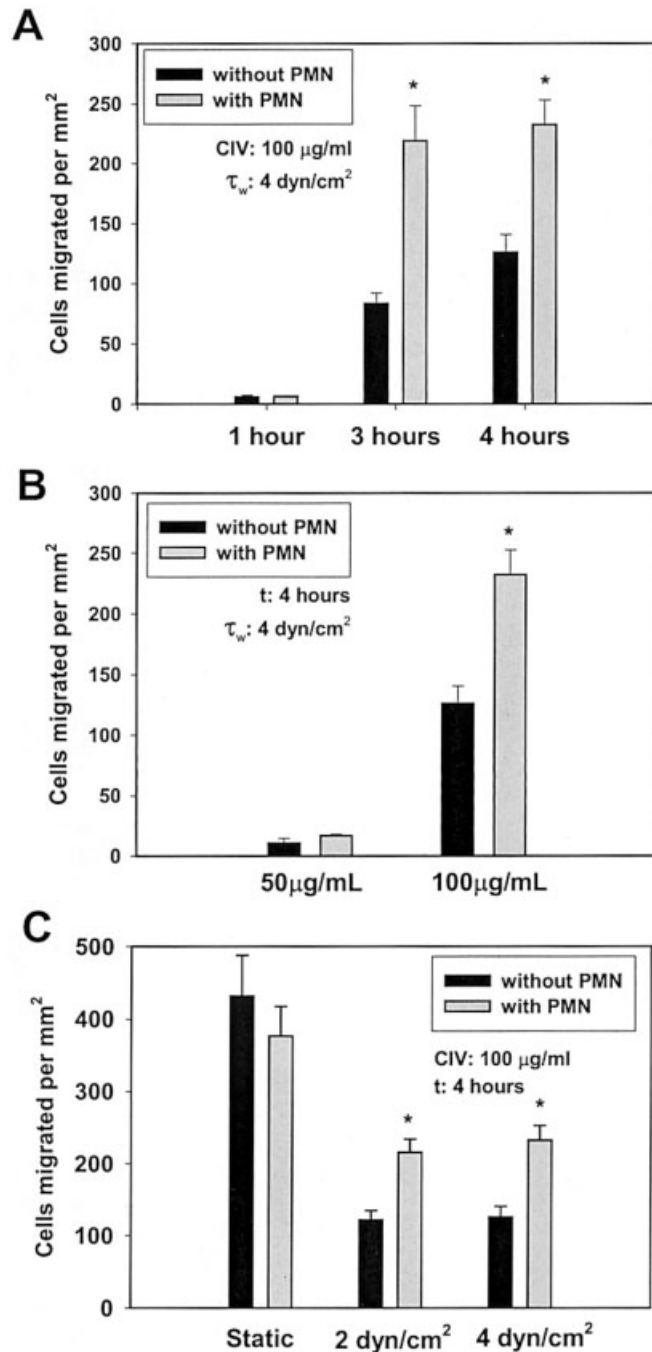


FIGURE 4 – Factors that influence C8161 cell chemotactic migration. (a) Time duration at 1, 3 and 4 hr; (b) Chemoattractant concentration at 50 and 100 µg/ml CIV and (c) Shear stress at 0, 2 and 4 dyn/cm². PMNs consistently increase C8161 chemotactic migration when each of these parameters is varied. * indicates $p=0.007$ with respect to the case under the same conditions but without PMNs (black bar to the left).

production by C8161 cells and PMNs, either with single cell type or 2 cell types in co-culture. ELISA was first used to detect background levels of secreted IL-8 in cell culture supernatant from 6 hr of culture. IL-8 production by C8161 cells was measured to be 250 ± 15 pg/ml and 65 ± 5 pg/ml by PMNs (Fig. 8a). Transwell co-culture samples were found to have larger IL-8 quantities (397 ± 20 pg/ml) that were above summed background levels. To more specifically identify which cells produced increased amounts

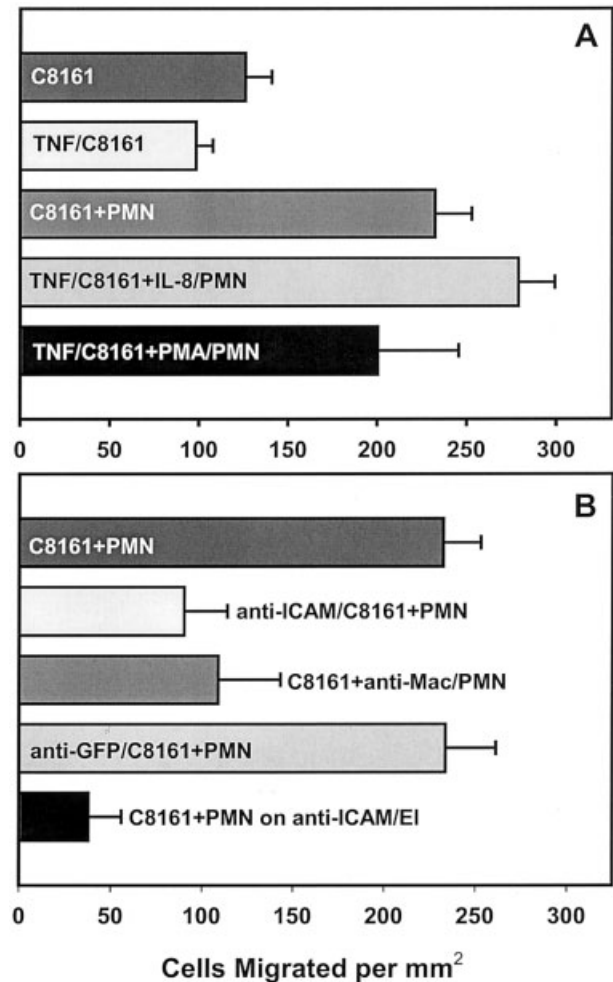


FIGURE 5 – (a) Migration of TNF α -activated C8161 cells (TNF/C8161) cells was found to be the same as unstimulated C8161 cells. In contrast, the migration of TNF/C8161 in the presence of IL-8-stimulated PMN (TNF/C8161+IL-8/PMN) increases C8161 migration by 20% compared to “C8161+PMN”. PMA-activated PMNs (TNF/C8161+PMA/PMN) do not increase melanoma cell migration significantly above “C8161+PMN”. (b) PMN-influenced C8161 cell (C8161+PMN) migration is significantly reduced by blocking either ICAM-1 on C8161 (anti-ICAM/C8161+PMN; $p<0.02$), Mac-1 on PMNs (C8161+anti-Mac/PMN; $p<0.04$) or ICAM-1 on the EI monolayer (C8161+PMN on anti-ICAM/EI; $p<0.001$). All p values are with respect to “C8161+PMN”. Nonspecific antibody treatment was found to have no effect on the tumor cells migration (anti-GFP/C8161+PMN). ICAM-1 antibody blocked C8161 cells were found to have similarly low migration to CIV, with or without PMNs (data not shown). All results were obtained under 100 µg/ml CIV and 4dyn/cm² shear stress over 4 hr.

of IL-8, cell lysates were then analyzed using Western blot. PMNs that had been Transwell co-cultured with C8161 cells contained more intracellular IL-8 as compared to PMNs cultured alone without C8161 cells (Fig. 8b). The lane labeled IL-8 indicates the positive control lane loaded with recombinant human IL-8. In addition, C8161 cells cultured with or without PMNs were shown to contain approximately the same amount of intracellular IL-8 (Fig. 8b). To verify these results and confirm this was not an artifact of extended co-culture, RT-PCR was performed after 6 hr of co-culture (Fig. 8c). The cDNA levels in the PMN samples increase where as the C8161 cDNA levels did not change. A change in IL-8 production by PMNs in presence or absence of

C8161 cells indicates that cell-cell communication may facilitate C8161 and PMN adhesion.

DISCUSSION

Cellular extravasation requires a coordination of many cellular events and processes. Development of an *in vitro* assay that characterizes these processes allows the ability to isolate and characterize factors that contribute to successful or inhibited extravasation. In the case of tumor cells, shear-resistant adhesion and migration are important steps in cancer metastasis formation. No

prior existing apparatus allowed both steps to be studied simultaneously. We have developed a novel flow-migration assay that allows cell-substrate adhesion and subsequent migration to be characterized and quantified in a controlled *in vitro* environment.

Endothelial cells transiently express ICAM-1, the ligand for β_2 -integrins. It is through this receptor-ligand interaction that firm adhesion of PMNs to the blood vessel wall is achieved. However, adhesion-mediated tumor cell arrest differs from the leukocyte adhesion and migration cascade.^{1,3,32} For example, tumor cells do not exhibit "leukocyte-like rolling" adhesive interaction with the endothelium for initial microvascular arrest (from cell lines of 6 different histological origins).³³ Several ligands for inducible endothelial adhesion molecules have been identified on various types of tumor cells.³⁴ Miele *et al.*³⁵ reported that a dose- and time-dependent increase in surface expression of ICAM-1 was found in human malignant melanoma cells treated with tumor necrosis factor- α (TNF- α). They also found that inhibiting ICAM-1 reduced melanoma lung metastasis *in vivo*. We have found very low levels of β_2 -integrins but relatively high levels of functional ICAM-1 on C8161 cells (Fig. 2a), as well as on another human melanoma cell line A2058 (data not shown). The relatively low surface expression of β_2 -integrins by C8161 cells makes melanoma cell adhesion to endothelial ICAM-1 improbable under physiological flow conditions. A potential mechanism for allowing melanoma cell adhesion to the endothelium under shear forces could be elicitation of β_2 -integrin expressing host cells in the blood circulation, *e.g.*, PMNs, to act as a binding mediator between the tumor cell and the endothelium. Several other studies have shown the ability of lymphocytes,³⁶ natural killer cells³⁷ and monocytes/macrophages³⁸⁻⁴⁰ in mediating tumor cell metastasis. There are also reports on PMN association with tumor cells *in vivo*.¹³⁻¹⁷

A monolayer of fibroblast L-cells transfected to express ICAM-1 adhesion molecules (termed EI cells) was used as an experimental model of endothelium. The level of ICAM-1 surface expression on the EI cells is comparable to IL-1 α -stimulated human umbilical vein endothelial cells (HUVEC),²⁹ thereby creating a stable adhesive substrate with a constant adhesion molecule expression. EI cells were selected over HUVEC or other endothelial cells because EI cells have a stable expression of ICAM-1 molecules, unlike HUVEC or other endothelial cells that may have transient changes in surface adhesion molecule expression. Since the EI cells were transfected with a stable expression of ICAM-1,²⁹ those EI cells were not stimulated by cytokines, such as IL-8 or TNF α ; therefore changes in ICAM-1 or β_2 expression were not tested in the present study. While the EI cells may not form a "cobblestone" patterned monolayer common to endothelial cells, they do form a stable monolayer that allows for cellular adhesion and requires migrating cells to crawl under to reach the micro-

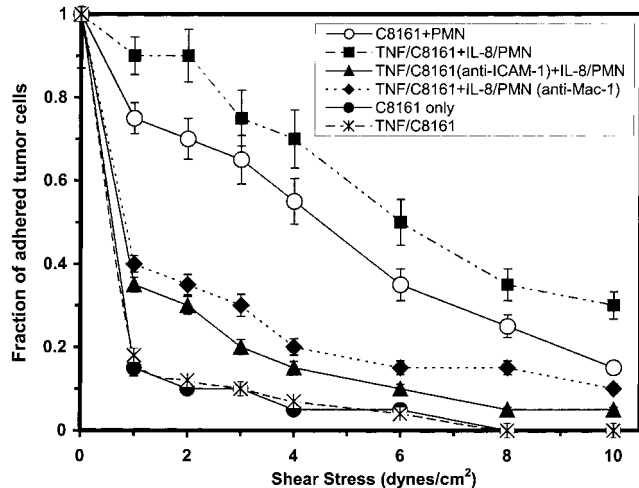
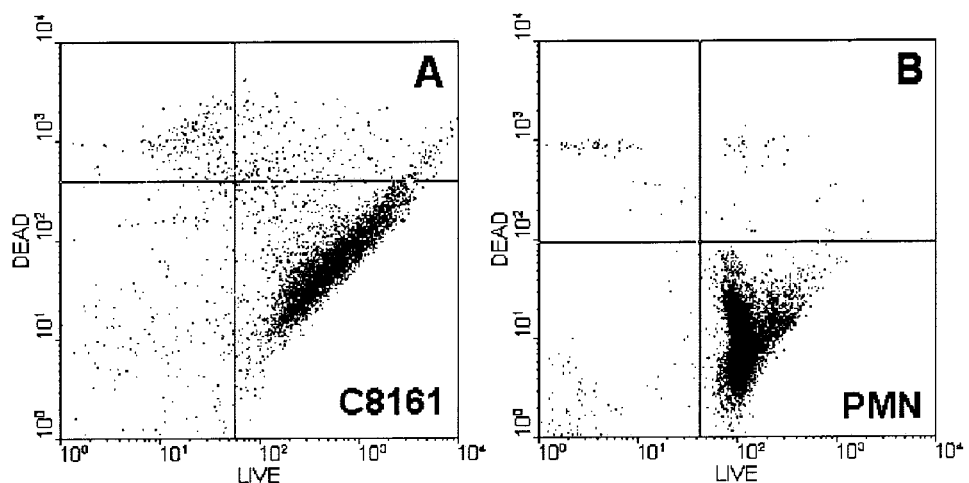


FIGURE 6—Adhesion assay examines heterotypic C8161-PMN aggregation that results in successful C8161 arrest on ICAM-1 expressing EI cell substrate under increasing shear stresses. Only the cases where a C8161 cell was paired with 1 or 2 PMNs were counted. Results are reported as the fraction of C8161 or C8161-PMN pairs that remain adhered under a given shear stress over 20 sec, normalized by the cell number initially adhered to the substrate under no-flow condition. Single type C8161 cells (closed circle) and TNF/C8161 cells (X) show the weakest adherence to EI cells at very low shear stresses. C8161-PMN cell pairs (open circle) are able to withstand significantly higher shear stresses. C8161 cell adhesion to EI substrate is further strengthened with cytokine activated C8161 and PMNs (TNF/C8161 and IL-8/PMN; closed square). The doublet adhesion is ablated when antibodies were used to block either ICAM-1 on TNF/C8161 cells (closed triangle) or Mac-1 on IL-8/PMNs (closed diamond). Lines connecting data points are included for clarity and do not imply curve fitting.

FIGURE 7—Representative flow cytometry dot plots of C8161 and PMN; stained red if they are dead (y-axis) and green if they are alive (x-axis). The fraction of cells in the lower right quadrant of each dot plot encompasses the live population. LIVE/DEAD viability data indicates that greater than 90% of C8161 cells and PMNs are alive after being Transwell co-cultured for 4 hr.



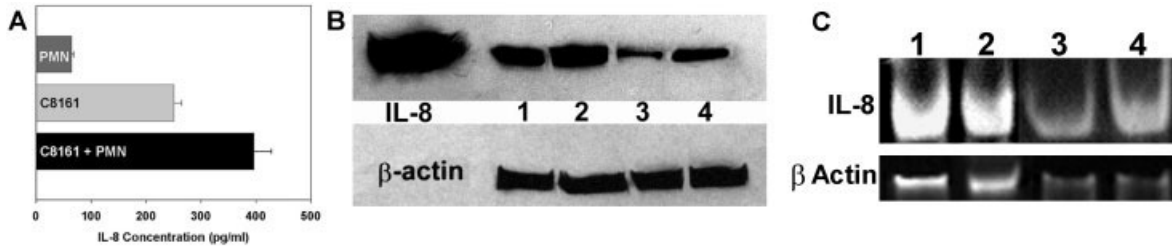


FIGURE 8 – Protein detection assays for IL-8 production by C8161 cells and PMNs. In general, cells were cultured in standard 6-well plates (for single-type cell culture) or Transwells (for 2 cell-type co-culture). (a) ELISA quantified background IL-8 production by PMNs to be 65 ± 5 pg/ml, by C8161 cells to be 250 ± 15 pg/ml and quantities above the sum (397 ± 20 pg/ml) were found in co-culture supernatant after 6 hr of incubation. (b) Cell lysates were analyzed using Western blot. C8161 cells had been Transwell co-cultured with PMNs. IL-8 levels in C8161 cells remain nearly constant when cultured alone (lane 1) or co-cultured with PMNs (lane 2). In contrast, IL-8 levels in PMNs significantly increase when they are co-cultured with C8161 cells (lane 4) compared to its culture alone case (lane 3). β -actin staining indicates lane loading and the lane labeled IL-8 is a standard positive control of recombinant human IL-8. (c) RT-PCR products from C8161 and PMN lysates after 6 hr of culture either alone or co-cultured together in Transwells. C8161 IL-8 cDNA level is similar in both cases, cultured alone (Lane 1) or cultured with PMNs (Lane 2). There is an increase in IL-8 cDNA level in PMNs after 6 hr of co-culture with C8161 melanoma cells (lane 4) as compared to PMNs cultured alone (lane 3). β -actin samples provide loading control.

porous filter. In shear stability tests, an EI monolayer has been exposed to shear stress (8 dyn/cm^2) over a period of 4 hr with little to no disruption to the monolayer (data not shown).

Migration of C8161 cells was characterized under static and shear conditions (4 dyn/cm^2). Under static conditions, C8161 cells do not have any difficulty migrating through the cell monolayer due to their invasive nature. Obviously, there are no fluid shear forces challenging the cell-substrate adhesion during tumor cell migration. In this scenario, the addition of PMNs to the tumor cell suspension does not result in any increase in C8161 migration. When a shear flow is present, C8161 migration decreases significantly, indicating that C8161 cell adhesion by itself to an ICAM-1 cellular monolayer is less efficient for successful cell migration under physiological shear forces. Melanocytes (NHEM) were also used as a nonmigratory control in the flow-migration chamber. They do express ICAM-1, but have not been tested for β_2 -integrin expression, as β_2 -integrin expression is common mainly to leukocytes and myeloid cells.⁴¹ Under static conditions, the NHEM cells did not migrate; they also failed to migrate in the presence of PMNs under shear conditions. While melanocytes appear to be able to adhere to collagen IV-bound substrate under static conditions,⁴² haptotaxis does not occur in the case of the flow-migration assay as CIV was strictly prepared for a soluble chemotactic gradient.

Shear stress in the flow system using both melanoma cells and PMNs appears to be comparable to the shear stress using just one cell type (e.g., melanoma cells only). Doubling of the cell number in the flow system does not seem to change the hydrodynamic effect on cell migration, which could be due to a large ratio of in flow ($\sim 50 \text{ ml}$) with respect to an increase in the number of cells ($\sim 5 \times 10^6$) that are in the system. There is a sharp drop in C8161 migration when a shear flow is present (e.g., $2\text{--}4 \text{ dyn/cm}^2$), indicating shear stress has significant effect on tumor cell adhesion and migration (Fig 4c). The insignificant difference between the cases of 2 and 4 dyn/cm^2 suggests that while the lower shear stress is sufficient to remove unattached cells, increasing the shear stress does not influence the migratory behavior of the cells.

Adding PMNs to the migration chamber regains a significant portion of C8161 migration under flow conditions. The increase in migration is even greater when both C8161 cells and PMNs are activated with inflammatory cytokines. For example, Figure 5a shows that the migration of TNF α -treated C8161 (TNF α /C8161) increases C8161 migration ($279 \pm 20 \text{ cells/mm}^2$) in the presence of IL-8-treated PMN (IL-8/PMN), which is 20% more than untreated C8161 cells and PMNs ($232 \pm 20 \text{ cells/mm}^2$). Cytokine treatment mimics an inflammatory state and exaggerates the potential for heterotypic aggregation between C8161 cells and PMNs. TNF α up-regulates ICAM-1 on C8161 cells, which increases the cellular

interactions *via* an ICAM-1/Mac-1 adhesion mechanism in the presence of IL-8/PMN. However, PMA-activated PMNs do not show as much influence on C8161 migration as the IL-8/PMN, suggesting less of a role for LFA-1 from PMNs in assisting tumor cell prolonged adhesion to the endothelium in a shear flow. This corresponds to a recent report that LFA-1 may play more of a role in “catching” rolling PMNs where as Mac-1 is primarily responsible for maintaining firm PMN-EC adhesion in a shear flow.¹¹ Blocking ICAM-1 on C8161 cells, even in the presence of PMNs, significantly reduces tumor cell migration. ICAM-1 blocked C8161 cells were found to have similar migration level with or without PMNs. Also blocking the Mac-1 on PMNs reduces C8161 migration. These findings strongly indicate that C8161 cells adhesion and migration under flow conditions is strongly mediated by PMNs *via* ICAM-1/Mac-1 adhesion interactions. An increase in melanoma cell migration as a result to the presence of PMNs has also been found in another metastatic melanoma cell line A2058 (data not shown).

Results also show that C8161-PMN aggregates adhere more strongly to ICAM-1 expressing EI cell substrate than single-type C8161 cells. PMNs that highly express Mac-1 and LFA-1 could possibly form a bridge and bind to both the ICAM-1 expressing C8161 and the EI cell monolayer. Antibody blocking of ICAM-1 on C8161 cells or blocking Mac-1 on PMNs dramatically reduces the C8161-PMN aggregation and subsequent C8161 adhesion to EI cells under increasing shear stresses, although ICAM-1 blocking appears to have more profound influence on C8161-PMN adhesion than Mac-1 blocking (Fig. 6). Another corroborating piece of data is that blocking the ICAM-1 on the EI monolayer, thereby preventing all adhesion to the monolayer, reduces C8161 migration more than 5-fold (Fig 5b). These assays give experimental evidence that implicate β_2 -integrin and ICAM-1 interactions in regulating C8161/PMN conjugate adhesion to the endothelial surface.

Melanoma cells and PMNs used in the assays were stimulated with proinflammatory cytokines (e.g., TNF α , IL-8, etc.) to stimulate adhesion molecule expression. These cytokines were not necessarily used to mimic a particular stage of the metastasis cascade, but C8161 cells and PMNs were found to secrete such cytokines and therefore have the potential to cross activate cells and promote cell-cell adhesion. Some other studies have also showed that IL-8 promotes the growth of some tumors and production is associated with metastatic potentials.²²

The nature of PMNs give cause to question their effect on C8161 cells in terms of cytotoxicity. Viability studies verify that within the time frame of the flow-migration assay (4 hr) the vast majority (greater than 90%) of C8161 cells and PMNs are alive when either cultured separately, together but separated by Trans-

well filters, in direct contact with each other, or suspended together in media. It is only in the case of direct contact culture for extended periods of time (20–24 hr) that there is significant cell death by C8161 cells. It is also in this extended time frame that PMNs no longer remain viable in culture.

PMN-C8161 attachment to the endothelium creates a microenvironment that is potentially self-stimulatory. Analysis of cytokine production by each of the 2 cell types during co-culture shows that proximity and cell-cell communication cause changes in IL-8 production. ELISA assays indicate that C8161 cells produce relatively high amounts of IL-8, but in contrast, PMNs do not when unstimulated. In Transwell co-culture with C8161 cells, PMNs significantly increase IL-8 production as determined by immunoblot. The trend of increasing amounts of IL-8 in PMNs during co-culture vs. constant expression by the C8161 cells was verified by RT-PCR. Because melanoma cells have not been found to express CXCR1 or CXCR2,⁴³ soluble IL-8 as released by C8161 cells and PMN would mainly have the environmental effect of stimulating nearby endothelium and leukocytes or potentially have an autocrine effect on PMNs, not melanoma cells themselves. Increased IL-8 secretion by PMNs in the presence of C8161 cells would potentially provide a mechanism by which C8161 cells and PMNs can influence their microenvironment. For example, we

have found that after 4 hr of contact-culture, PMNs express Mac-1 significantly above basal levels (data not shown). Recent reports confirm that β_2 -integrin expression on PMNs is increased by IL-8.⁴⁴ ICAM-1 expression on C8161 cells after 4 hr of co-culture also increased but to a lesser extent (data not shown). Up-regulation of adhesion molecules in response to cytokine signaling is important in initiating C8161-PMN aggregation and tumor cell adhesion to an endothelial surface under flow conditions, which directly affect subsequent tumor cell chemotactic migration during the extravasation process. Studies to characterize the adhesion of tumor cells to PMNs on a substrate are underway and will help delineate the role of PMNs in aiding adhesion vs. the role they have in tumor cell migration and metastasis.

ACKNOWLEDGEMENTS

The authors appreciate the support of the Penn State General Clinical Research Center (GCRC) staff that provided nursing care and performed the ELISA assays. The GCRC is supported by NIH Grant M01-RR-10732. We also thank Dr. S. Simon and Dr. D. Welch for providing cell lines to this project and Dr. L. Hodgson and Dr. A. Henderson for assistance with the immunoblot and RT-PCR.

REFERENCES

- Chambers AF, MacDonald IC, Schmidt EE, Morris VL, Groom AC. Clinical targets for anti-metastasis therapy. *Adv Cancer Res* 2000;79: 91–121.
- Liotta LA. Cancer cell invasion and metastasis. *Sci Am* 1992;266: 54–63.
- Scherbarth S, Orr FW. Intravital video microscopic evidence for regulation of metastasis by the hepatic microvasculature: Effects of interleukin-1 α on metastasis and the location of B16F1 melanoma cell arrest. *Cancer Res* 1997;57:4105–10.
- Zetter BR. Adhesion molecules in tumor metastasis. *Semin Cancer Biol* 1993;4:219–29.
- Harlan JM, Liu DY, eds. Adhesion: its role in inflammatory disease. New York: WH Freeman, 1992.
- Lauffenburger D, Linderman JJ. Receptors: Models for binding, trafficking, and signaling. New York: Oxford, 1983.
- Smith CW, Marlin SD, Rothlein R, Toman C, Anderson DC. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils *in vitro*. *J Clin Invest* 1989;83: 2008–17.
- Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 1991;65:859–73.
- Lay K, Zakrzewicz A, Hanski C, Stoolman LM, Kansas GS. Sialylated O-Glycans and L-selectin sequentially mediate myeloid cell rolling *in vivo*. *Blood* 1995;85:3727–35.
- Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994;76:301–14.
- Hentzen ER, Neelamegham S, Kansas GS, Benanti JA, McIntire LV, Smith CW, Simon SI. Sequential binding of CD11a/CD18 and CD11b/CD18 defines neutrophil capture and stable adhesion to intercellular adhesion molecule-1. *Blood* 2000;95:911–20.
- Neelamegham S, Taylor AD, Burns AR, Smith CW, Simon SI. Hydrodynamic shear shows distinct roles for LFA-1 and MAC-1 in neutrophil adhesion to intercellular adhesion molecule-1. *Blood* 1998; 92:1626–38.
- Aeed PA, Nakajima M, Welch DR. The role of polymorphonuclear leukocytes (PMN) on the growth and metastatic potential of 13762NF mammary adenocarcinoma cells. *Int J Cancer* 1988;42:748–59.
- Welch DR, Schissel DJ, Howrey RP, Aeed PA. Tumor-elicited polymorphonuclear cells, in contrast to “normal” circulating polymorphonuclear cells, stimulate invasive and metastatic potentials of rat mammary adenocarcinoma cells. *Proc Natl Acad Sci USA* 1989;86:5859–63.
- Wu QD, Wang JH, Condran C, Bouchier-Hayer D, Redmond HP. Human neutrophils facilitate tumor cell transendothelial migration. *Am J Physiol Cell Physiol* 2001;280:C814–22.
- Starkey JR, Liggitt HD, Jones W, Hosick HL. Influence of migratory blood cells on the attachment of tumor cells to vascular endothelium. *Int J Cancer* 1984;34:535–43.
- Crissman JD, Hatfield J, Schaldenbrand M, Sloane BF, Honn KV. Arrest and extravasation of B16 amelanotic melanoma in murine lungs: a light and EM study. *Lab Invest* 1985;53:470–8.
- Zachariae COC, Thestrup-Pedersen K, Matsushima K. Expression and secretion of leukocyte chemotactic cytokines by normal human melanocytes and melanoma cells. *J Invest Dermatol* 1991;97:593–9.
- Smith WB, Gamble JR, Clark-Lewis I, Vadas MA. Interleukin-8 induces neutrophil transendothelial migration. *Immunology* 1991;72: 65–72.
- Yamamura K, Kibbey MC, Kleinman HK. Melanoma cells selected for adhesion to laminin peptides have different malignant properties. *Cancer Res* 1993;53:423–8.
- Larsen CG, Anderson AO, Appella E, Oppenheim JJ. The neutrophil-activating protein (NAP-1) is also chemotactic for T-lymphocytes. *Science* 1989;243:1461–6.
- Luca M, Huang S, Gershenwald JE, Singh RK, Reich R, Bar-Eli M. Expression of Interleukin-8 by Human melanoma Cells Up-Regulates MMP-2 Activity and Increases Tumor Growth and Metastasis. *Am J Pathol* 1997;151:1105–13.
- Cao J, Donell B, Deaver DR, Lawrence MB, Dong C. *In vitro* side-view technique and analysis of human T-leukemic cell adhesion to ICAM-1 in shear flow. *Microvasc Res* 1998;55:124–37.
- Lawrence MB, McIntire LV, Eskin SG. Effect of flow on polymorphonuclear leukocyte/endothelial cell adhesion. *Blood* 1987;70: 1284–90.
- Aznavorian S, Stracke ML, Krutzsch HC, Schiffmann E, Liotta LA. Signal transduction for chemotaxis and haptotaxis by matrix molecules in tumor cells. *J Cell Biol* 1990;110:1427–38.
- Dong C, Slattery MJ, Rank BM, You J. *In Vitro* characterization and micromechanics of tumor cell chemotactic protrusion, locomotion and extravasation. *Ann Biomed Eng* 2002;30:344–55.
- Welch DR, Bisi JE, Miller BE, Conaway D, Seftor EA, Yohem KH, Gilmore LB, Seftor REB, Nakajima M, Hendrix MJC. Characterization of a Highly Invasive and Spontaneously Metastatic Human Malignant Melanoma Cell Line. *Int J Cancer* 1991;47:227–37.
- Hodgson L, Qiu W, Dong C, Henderson AJ. Use of green fluorescent protein-conjugated beta-actin as a novel molecular marker for *in vitro* tumor cell chemotaxis assay. *Biotechnol Prog* 2000;16:1106–14.
- Gopalan PK, Smith CW, Lu H, Berg E, McIntire LV, Simon SI. PMN CD18-dependent arrest on ICAM-1 in shear flow can be activated through L-selectin. *J Immunol* 1997;158:367–75.
- Hodgson L, Dong C. $[Ca^{2+}]_i$ as a potential down-regulator of $\alpha_5\beta_1$ -integrin mediated A2058 tumor cell migration to type IV collagen. *Am J Physiol Cell Physiol* 2001;281:C106–13.
- You J, Mastro AM, Dong C. Application of the dual-micropipet technique to the measurement of tumor cell locomotion. *Exp Cell Res* 1999;248:160–71.
- Giavazzi R, Foppolo M, Dossi R, Remuzzi A. Rolling and adhesion of human tumor cells on vascular endothelium under physiological flow conditions. *J Clin Invest* 1993;92:3038–44.
- Thorlacius H, Pricto J, Raud J, Gautam N, Patarroyo M, Hedqvist P, Lindbom L. Tumor cell arrest in the microcirculation: Lack of evi-

- dence for a leukocyte-like rolling adhesive interaction with vascular endothelium *in vivo*. *Clin Immunol Immunopathol* 1997;83:68–76.
34. Yamada KM. Introduction: Adhesion molecules in cancer. Part I. *Semin Cancer Biol* 1993;4:215–8.
 35. Miele ME, Bennett CF, Miller BE, Welch DR. Enhanced metastatic ability of TNF-treated malignant melanoma cells is reduced by intercellular adhesion molecule-1 (ICAM-1) antisense oligonucleotides. *Exp Cell Res* 1994;214:231–41.
 36. Kripke MI. Ultraviolet radiation and immunology: something new under the sun—Presidential address. *Cancer Res* 1994;54:6102–5.
 37. Hanna N. The role of natural killer cells in the control of tumor growth and metastasis. *Biochim Biophys Acta* 1985;780:213–26.
 38. Key ME. Macrophages in cancer metastasis and their relevance to metastatic growth. *Cancer Metastasis Rev* 1983;2:75–88.
 39. Mantovani A. Tumor-associated macrophages in neoplastic progression: a paradigm for the *in vivo* function of chemokines. *Lab Invest* 1994;71:5–16.
 40. van Netten JP, Ashmead BJ, Parker RL, Thornton IG, Fletcher C, Cavers D, Coy P, Brigden ML. Macrophage-tumor cell association: a factor in metastasis of breast cancer? *J Leukoc Biol* 1993;54:360–2.
 41. Yohn JJ, Critelli M, Lyons MB, Norris DA. Modulation of melanocyte intercellular adhesion molecule expression by immune cytokines. *J Invest Dermatol* 1990;90:233–7.
 42. Neitmann M, Alexander M, Brinckmann J, Schlenke P, Tronnier M. Attachment and chemotaxis of melanocytes after ultraviolet irradiation *in vitro*. *Br J Dermatol* 1999;141:794–801.
 43. Müller A, Homet B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001;410:50–6.
 44. Takami M, Terry V, Petruzzelli L. Signaling pathways involved in IL-8 dependent activation of adhesion through Mac-1. *J Immunol* 2002;168:4559–66.